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Visualisation of the obligate hydrocarbonoclastic bacteria *Polycyclovorans algicola* and *Algiphilus aromaticivorans* in co-cultures with micro-algae by CARD-FISH

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Abstract

Some studies have described the isolation and 16S rRNA gene sequence-based identification of hydrocarbon-degrading bacteria living associated with marine eukaryotic phytoplankton, and thus far the direct visual observation of these bacteria on micro-algal cell surfaces ('phycosphere') has not yet been reported. Here, we developed two new 16S rRNA-targeted oligonucleotide probes, PCY223 and ALGAR209, to respectively detect and enumerate the obligate hydrocarbonoclastic bacteria *Polycyclovorans algicola* and *Algiphilus aromaticivorans* by Catalyzed Reporter Deposition Fluorescence *in situ* Hybridisation (CARD-FISH). To enhance the hybridization specificity with the ALGAR209 probe, a competitor probe was developed. These probes were tested and optimized using pure cultures, and then used in enrichment experiments with laboratory cultures of micro-algae exposed to phenanthrene, and with coastal water enriched with crude oil. Microscopic analysis revealed these bacteria are found in culture with the micro-algal cells, some of which were found attached to algal cells, and whose abundance increased after phenanthrene or crude oil enrichment. These new probes are a valuable tool for identifying and studying the ecology of *P. algicola* and *A. aromaticivorans* in laboratory and field samples of micro-algae, as well as opening new fields of research that could harness their ability to enhance the bioremediation of contaminated sites.

Introduction

Obligate hydrocarbonoclastic bacteria (OHCB) are specialists with respect to their ability to utilise hydrocarbons almost exclusively as a carbon and energy source. The occurrence of these fastidious hydrocarbon degraders appears to be solely confined to the marine environment since, to the best of our knowledge, they are found nowhere else on earth. They play an important role in the removal and mineralization of hydrocarbon pollutants in the oceans and seas, as is evidenced in the wealth of reports documenting their enrichment from near undetectable abundance levels (<0.1%) to constituting up to 90% of the total bacterial community at oil-impacted sites (Head *et al*., 2006; Yakimov *et al*., 2007). With the exception of *Planomicrobium alkanoclasticum* (a Gram-positive Firmicute), most of the recognised OHCB are classified within the order *Oceanospirillales* of the class *Gammaproteobacteria*, and comprise members of the genera *Alcanivorax*, *Oleiphilus*, *Oleispira*, *Oleibacter* and *Thalassolitus* that specialize in the degradation of linear or branched saturated hydrocarbons, whereas two genera, *Cycloclasticus* and *Neptunomonas*, specialize in the degradation of polycyclic aromatic hydrocarbons (PAHs). The detection, identification and monitoring of these types of organisms in a highly-resolved manner and within samples collected *in situ* is highly advantageous to expand our knowledge on their geography and ecology.

The phycosphere of marine eukaryotic phytoplankton is an underexplored biotope in the ocean where OHCB and 'generalist' oil-degrading bacteria have been identified. Hydrocarbon-degrading bacteria, including some comprising novel taxa, have been isolated from all three major phytoplankton lineages of dinoflagellates, diatoms and coccolithophores (Green *et al.*, 2004; Green *et al*., 2006; Green *et al*., 2015; Gutierrez *et al.*, 2012a,b; 2013; 2014). This algal-bacterial association raises important questions regarding the interplay and ecology between these organisms, what contribution this has to the biodegradation of hydrocarbons in the ocean, and to their evolutionary genesis. Enhanced degradation of hydrocarbons has been demonstrated when bacteria and phytoplankton coexist (e.g., Abed and Köster, 2005; Warshawsky *et al.*, 2007), and

more recent work has shown that the bacterial community associated with phytoplankton may be better tuned to respond to and degrade hydrocarbons when challenged with crude oil (Mishamandani *et al*., 2016; Thompson *et al*., 2017). Whether through biogenic synthesis (Andelman and Suess, 1970; Gunnison and Alexander, 1975; Zelibor *et al.*, 1988; Marlowe *et al.*, 1984; Shaw *et al.*, 2010; Exton *et al.*, 2012) or adsorption of hydrocarbon molecules from the surrounding seawater (Mallet and Sarfou, 1964; Andelman and Suess 1970), phytoplankton cells can be regarded as a "hot spot" to which hydrocarbon-degrading bacteria are attracted to and, through evolution, have settled into a state of co-existence.

Two species of known OHCB representing a novel genus (*Polycyclovorans*) and family (*Algiphilus*) were originally isolated from eukaryotic phytoplankton, but are poorly represented in 16S rRNA gene sequence databases (Gutierrez *et al*., 2012a; Gutierrez *et al*., 2013). The type species of these two genera, *Algiphilus aromaticivorans* and *Polycyclovorans algicola*, were respectively isolated from the dinoflagellate *Lingulodinium polyedrum* (Gutierrez *et al*., 2012a) and the cosmopolitan marine diatom *Skeletonema costatum* (Gutierrez *et al*., 2013). Both organisms belong within the order *Xanthomonadales* and found to exhibit a narrow nutritional spectrum, preferring to utilise mono- and polycyclic aromatic hydrocarbons (PAHs) and some *n*-alkanes and organic acids. Using quantitative PCR primers designed to target the 16S rRNA gene of *P. algicola* strain showed that this organism is also associated with other species of marine diatoms and also dinoflagellates (Gutierrez *et al*., 2013) and coccolithophores (unpublished data). These organisms were identified in these studies because they were targeted based on their ability to utilise hydrocarbons as a sole source of carbon and energy, and because they were searched for in the right place – i.e. in cultures of marine eukaryotic phytoplankton. When isolating for these types of organisms in seawater samples, in the absence of methods that could allow researchers to discern if they are free living or associated with particulate matter (e.g. transparent exopolymer, marine snow, phytoplankton, etc.), the common mistake is to assume these organisms exist in a free-living state. In order to improve our understanding on the ecology of hydrocarbon-degrading

bacteria found associated with phytoplankton and their role in the event of oil contamination in the marine environment, it is imperative to have a method that can identify and monitor the abundance of these organisms in environmental samples.

CARD-FISH (Catalyzed Reporter Deposition Fluorescence *in situ* Hybridisation) is an effective technique allowing phylogenetic identification, enumeration, and direct spatial visualization of microorganisms in their natural environment by targeting the ribosomal RNA (rRNA) of bacterial cells of interest (Pernthaler *et al.,* 2002; Schönhuber *et al.,* 1999). CARD-FISH is an *in situ* amplification method utilizing horseradish peroxidase, which enhances bacterial cell detection over standard FISH protocols that can be several orders of magnitude less sensitive. The value of this method is in its ability to allow the observer to visualize the cells being targeted by the probe(s) in natural (*in situ*) environmental samples. Genus-level oligonucleotide probes were previously developed to identify members of the OHCB *Alcanivorax* (Syutsubo *et al*., 2001), *Cycloclasticus* (Maruyama *et al*., 2003) and *Marinobacter* (Mckay *et al*., 2016) by conventional FISH. CARD-FISH has been used to detect epiphytic bacteria on algae (Mayali *et al.,* 2011; Simon *et al.,* 2002; Tujula *et al.,* 2005), although this method has not, hitherto, been used to explore the occurrence of hydrocarbon-degrading bacteria associated with eukaryotic phytoplankton. In this study, we developed and optimized new probe sets to detect *A. aromaticivorans* and *P. algicola* by CARD-FISH. We then used these probes to examine the presence and spatial association of these organisms with laboratory cultures of phytoplankton.

Materials and methods

Oligonucleotide probe design

Oligonucleotide probes targeting *Polycyclovorans algicola* or *Algiphilus aromaticivorans* were designed against current 16S rRNA gene sequence databases. Using the Probe Design tool of Arb v104 (Pruesse *et al*., 2007), probe candidates were selected based on specific targeting of the 16S rRNA sequences for these two species. Probe candidates were analysed using the ProbeCheck

server (Loy *et al*., 2008), the Ribosomal Database Project's Probe Match tool (Cole *et al*., 2009), and the ARB-Silva TestProbe tool (Ludwig *et al*., 2004) to evaluate their *in silico* specificity and coverage. From this, one probe sequence, PCY223 (5'-TCA GAC ATA GGC TCC TCC AA-3'; 20-mer) was selected for *Polycyclovorans algicola*, and the probe sequence ALGAR209 (5'-CCT CCA GCG TGA GGT CCG-3'; 18-mer) was selected for *Algiphilus aromaticivorans*. A competitor probe, c1ALGAR209 (5'-CCT CCA GCG CGA GGT CCG-3'; 18-mer), was designed to improve signal intensities in hybridisations targeting *Algiphilus aromaticivorans*. Table 1 summarizes the probes that were developed in this study. *In silico* analysis of both probes using the SILVA 16S rRNA gene database revealed 13 uncultivated clone sequences with 100% sequence match to the PCY223 probe, and 219 sequences with a 1-basepair non-weighted mismatch. For the ALGAR209 probe, however, only 18 sequences with a 1-basepair non-weighted mismatch were found to uncultivated clones.

Oligonucleotide probe optimization

In order to test and optimize the CARD-FISH probes, pure cultures of *Polycyclovorans algicola* and *Algiphilus aromaticivorans* were grown in a defined synthetic seawater medium, ONR7a (Dyksterhouse *et al*., 1995), that was supplemented after steam-sterilization with filter-sterile (0.2 µm) trace elements and vitamins to final concentrations as previously described (Blackburn *et al*., 1989). Cells were permeabilized by incubation in lysozyme buffer (1.355×10^6 U ml^{-1} lysozyme, 50 mM EDTA [pH 8.0], 300 mM Tris-HCl [pH 8.0]) at 37°C for 2 h. The slides were washed in water for 1 min, incubated in 0.01M HCl for 10 min to bleach endogenous peroxidases, and then washed again in water for 1 min and air dried.

148 **Table 1.** 16S rRNA-targeted oligonucleotide FISH probes developed in this study.

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Probe name	Probe full name[1]	Sequence 5'-3'	Coverage[2]	Specificity[2]	Target organism	Optimal [FA]
PCY223	S-S-PCY223-a-A-20	TCA GAC ATA GGC TCC TCC AA	Genus *Polycyclovorans* (22%)	0	*Polycyclovorans algicola* (FJ176554)	55-60%
ALGAR209	S-S-ALGAR209-a-A-18	CCT CCA GCG TGA GGT CCG	Genus *Algiphilus* (33%)	0	*Algiphilus aromaticivorans* (DQ486493)	35-40%
c1ALGAR209	S-S-c1ALGAR209-a-A-18	CCT CCA GCG CGA GGT CCG			Competitor for ALGAR209	

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151 [1]According to Alm et al. (1996)

152 [2]*In silico* coverage (% of target taxon) and specificity (number of matched sequences outside the target taxon for cultivated strains) were evaluated

153 using the TestProbe tool of ARB Silva with default settings (0 mismatches) and database SSU132RefNR.

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Optimal conditions for hybridization with these probes were determined by performing multiple hybridisations using increasing formamide (FA) concentrations from 0% to 70%. The competitor probe c1ALGAR209 was always used in equimolar concentration with its respective probe ALGAR209. Hybridization buffers contained 900 mM NaCl, 20mM Tris-HCl (pH 8), 10% (w/v) dextran sulfate (Sigma-Alrich), 0.01% (w/v) sodium dodecyl sulphate and 10% blocking solution. The blocking solution consisted of 10% blocking reagent (Perkin Elmer) and maleic acid buffer (1.16% [w/v] maleic acid, 150 mM NaCl, pH 7.5). The washing buffer consisted of NaCl (variable concentration to maintain hybridization stringency), 20 mM Tris-HCl (pH 8), 5 mM EDTA (pH 8) and 0.01% (w/v) SDS. Excess washing buffer was removed with blotting paper. The samples were incubated for 20 min with PBS (~10 ml) and then at 46°C for 30 min with 1 part fluorescently-labelled tyramide and 499 parts amplification buffer (10% [v/v] blocking solution, 2 M NaCl, 10% [w/v] dextran sulfate, 0.0015% [v/v] H_2O_2, 100% [v/v] PBS [pH 7.3]). Samples were then washed in PBS (pH 7.3) for 20 min, followed by three washes with water for 1 min and then 96% ethanol for 3 sec before air-drying. The samples were stored at -20°C for subsequent microscopic analysis.

Samples were covered in mountant (80% [v/v] Citifluor, 14% [v/v] Vectashield, 1 μg ml^{-1} DAPI in 100% PBS [pH 9]) for visualisation using a Zeiss (Axio Scope.A1) epifluorescence microscope equipped with a Zeiss digital fluorescence imaging camera (AxioCam MRm). Amplified signal intensities were quantified using Zeiss Zen-Blue (2012) imaging analysis software. Six to eight fields of view (~300 – 500 bacterial cells) were counted for fluorescence intensity maximum quantification.

Polycyclovorans algicola and *Algiphilus aromaticivorans* enrichment setup using laboratory micro-algal cultures

To directly visualize the association of *Polycyclovorans algicola* strain TG408 and *Algiphilus aromaticivorans* strain DG125 with micro-algal cells, and to assess their response when exposed to phenanthrene, a time-series enrichment incubation was setup. The marine algal strains used were non-axenic laboratory cultures of the cosmopolitan diatom *S. costatum* strain CCAP 1077/1C and the dinoflagellate *Lingulodinium polyedrum* strain CCAP 1121/2. These micro-algae were used because they were the source in the original isolation of *Polycyclovorans algicola* strain TG408 and *Algiphilus aromaticivorans* strain DG125, respectively (Gutierrez *et al*., 2012a; Gutierrez *et al*., 2013). Both strains were purchased from the Culture Collection of Algae and Protozoa (CCAP; Oban, Scotland) and maintained in f/2 + Si algal medium (Guillard, 1975; Guillard and Ryther, 1962) in a temperature-controlled 12°C illuminated incubator.

Enrichments were prepared using twelve steam-sterilised 100-ml glass Erlenmeyer flasks capped with cotton bungs, each containing 49 ml of f/2 + Si medium. Six of the flasks were supplemented with phenanthrene (200 μg per flask) using acetone as carrier, and the acetone allowed to volatilize overnight prior to adding the f/2 + Si medium. The phenanthrene was allowed to equilibrate with the f/2 + Si medium for 3 days at 16°C prior to inoculation. Three of these flasks were inoculated with 1 ml of an *S. costatum* culture grown to the exponential phase, whereas the other three flasks were inoculated in the same way with *L. polyedrum*. The other six flasks containing f/2 + Si medium, but no phenanthrene, were inoculated in the same way – three with *S. costatum* and three with *L. polyedrum*. All twelve flasks were incubated in a temperature-controlled illuminated incubator with gentle shaking, at 16°C, with a 12:12 light/dark cycle and at a photon flux density of 150 μmol s^{-1} m^{-2}). At days 0, 3, 5 and 8, sub-samples (0.5 ml) were taken from each flask and fixation performed by mixing with 3% (v/v) paraformaldehyde for 3 hours at 4°C on Isopore polycarbonate filters (0.22 μm). Filters were washed three times with sterile ice-cold 1× PBS, air-dried and stored at 4°C for subsequent analysis within 48 hours.

CARD-FISH analysis of *Polyclovorans algicola* and *Algiphilus aromaticivorans* enrichments with micro-algae

Sub-samples fixed onto polycarbonate filters from the time-series incubations enriched with or without phenanthrene were dipped into 0.2% (w/v) agarose, then mounted onto glass slides and air dried following standard methods (Pernthaler *et al*., 2002). CARD-FISH was performed directly on the agarose-embedded samples using the optimized protocol developed for the PCY223 or ALGAR209 probes at a FA concentration of, respectively, 55% or 40%, with the exception that 50–100 µl of hybridization buffer were used. All hybridisations were counterstained with DAPI following standard methods (Porter and Feig, 1980) prior to visualization under the epifluorescence microscope and analysed using Zeiss Zen-Blue image processing software, as described above.

Field sample collection and CARD-FISH analysis of *Polyclovorans algicola* and *Algiphilus aromaticivorans* in natural communities of phytoplankton

During a sampling operation aboard *RV Serpula* on 24 May 2013 to a sampling station in the lower basin of Loch Creran (56º30.820N, 5º22.817W) located on the west coast of Scotland, a phytoplankton net (50-60 µm mesh size) was trawled near the sea surface (1–2 m depth) for several minutes to collect the phytoplankton community. The phytoplankton net sample was passaged through a 125-µm metal mesh filter to remove grazers, washed by gentle centrifugation with sterile synthetic seawater, and then stored at 4°C and used within 24 hours for the preparation of a crude oil enrichment experiment, as previously described (Thompson *et al*., 2017). Briefly, 500 ml of the washed phytoplankton sample, with its associated bacterial community, was added to 10 litres of filtered (0.2 µm) seawater. This was used to set up a crude oil enrichment by adding 350 ml of the phytoplankton suspension into 1-litre Erlenmeyer flasks (performed in triplicate) and then amended with filter-sterilised (0.2 µm) Heidrun crude oil (ca.

1% v/v final concentration). A control treatment was set up in the exact same way, with the exception that the oil was not added. The flasks were incubated in a temperature-controlled 15°C illuminated incubator with a 12:12 light/dark cycle (photon flux density ~100 μmol s^{-1} m^{-2}). Samples for CARD-FISH analysis with probes PCY223 and ALGAR209 were taken at days 2 and 5. The dynamics of the phytoplankton and bacterial community in these treatments, and results for hydrocarbon analysis, are reported elsewhere (Thompson *et al*., 2017).

Probes, chemicals and reagents

Probes ALGAR209 and PCY223 for CARD-FISH labelled with HRP at the 5' end were purchased from Biomers and Thermo Fisher Biopolymers, respectively. The competitor probe (c1ALGAR209) without HRP labelling, was purchased from Integrated DNA Technologies. Cy3-labelled tyramides were purchased from Perkin Elmer. The phenanthrene (>99.5% purity) was purchased from Sigma-Aldrich. All other chemicals were of molecular biology or HPLC grade.

Results and discussion

***P. algicola* and *A. aromaticivorans* phylogeny and probe design**

At the time of analysis, the PCY223 probe sequence was found to be 100% complementary to 43 *Gammaproteobacteria* and to one unclassified *Proteobacteria*. These 43 *Gammaproteobacteria* are affiliated to the family *Sinobacteriaceae* and comprise uncultivated clones, with the exception of 1 unclassified isolate designated strain DG1192 (EU052753) and the target organism *P. algicola* TG408. A previously reported (Gutierrez *et al*., 2013), 16S rRNA gene sequence analysis showed strain TG408 is affiliated with the family *Sinobacteraceae*, and further supported by the strain's DNA G+C content of 64.3 mol%, which is similar to that of most members of the *Sinobacteraceae* family (60–65 mol%).

Analysis of the ALGAR209 probe sequence showed it was 100% complementary to solely the target organism, *A. aromaticivorans* DG1253; it showed no match to any other cultivated strains or uncultivated clones or sequences. The phylogenetic position of this organism lies closest to the family *Sinobacteraceae*, but is poorly supported (low bootstrap value of <50%) and indicates the strain is only moderately affiliated with this family (confidence threshold of <89%) (Gutierrez *et al*., 2012a). Hence, the phylogenetic position of the strain in the order *Xanthomonadales* is unique, distinct, and probe ALGAR209 was successfully designed to target solely this organism.

PCY223 and ALGAR209 probe optimization

The hybridization conditions for probes PCY223 and ALGAR209 were optimised by hybridisations with the target strain (Figure 1A and 1B). As expected, the fluorescence intensity for the two target strains was greater than that employing the nonsense probe over a wide range of formamide concentrations. In the case of probe PCY223, fluorescence signal intensities dramatically decreased at formamide concentrations higher than 60%. The formamide series experiments indicated that concentrations of 40-60% would be suitable for use with this probe. Whilst a 60% formamide concentration appears as the ideal stringency for probe PCY223, this concentration, however, is at the maximal point where specificity of the probe dramatically decreases with higher formamide concentrations. Therefore, 55% was selected as the empirically optimized formamide concentration for this probe.

For the formamide series experiments with probe ALGAR209, highest fluorescence signal intensities occurred with formamide concentrations between 20-40%. Decay of the fluorescence signal occurred rapidly with formamide concentrations <20% and >40%. An optimised formamide concentration of 40% was, hence, selected that would be at the high end of stringency and still produce relatively strong fluorescence signals. Use of these optimised formamide concentrations for probe PCY223 (55% FA) and for ALGAR209 (40% FA) were

tested in subsequent hybridization experiments using pure cultures for each of their target strains – respectively, *P. algicola* TG408 (Fig. 1C) and *A. aromaticivorans* DG1253 (Fig. 1D) – and as expected showed strong cell signal intensities for both probes.

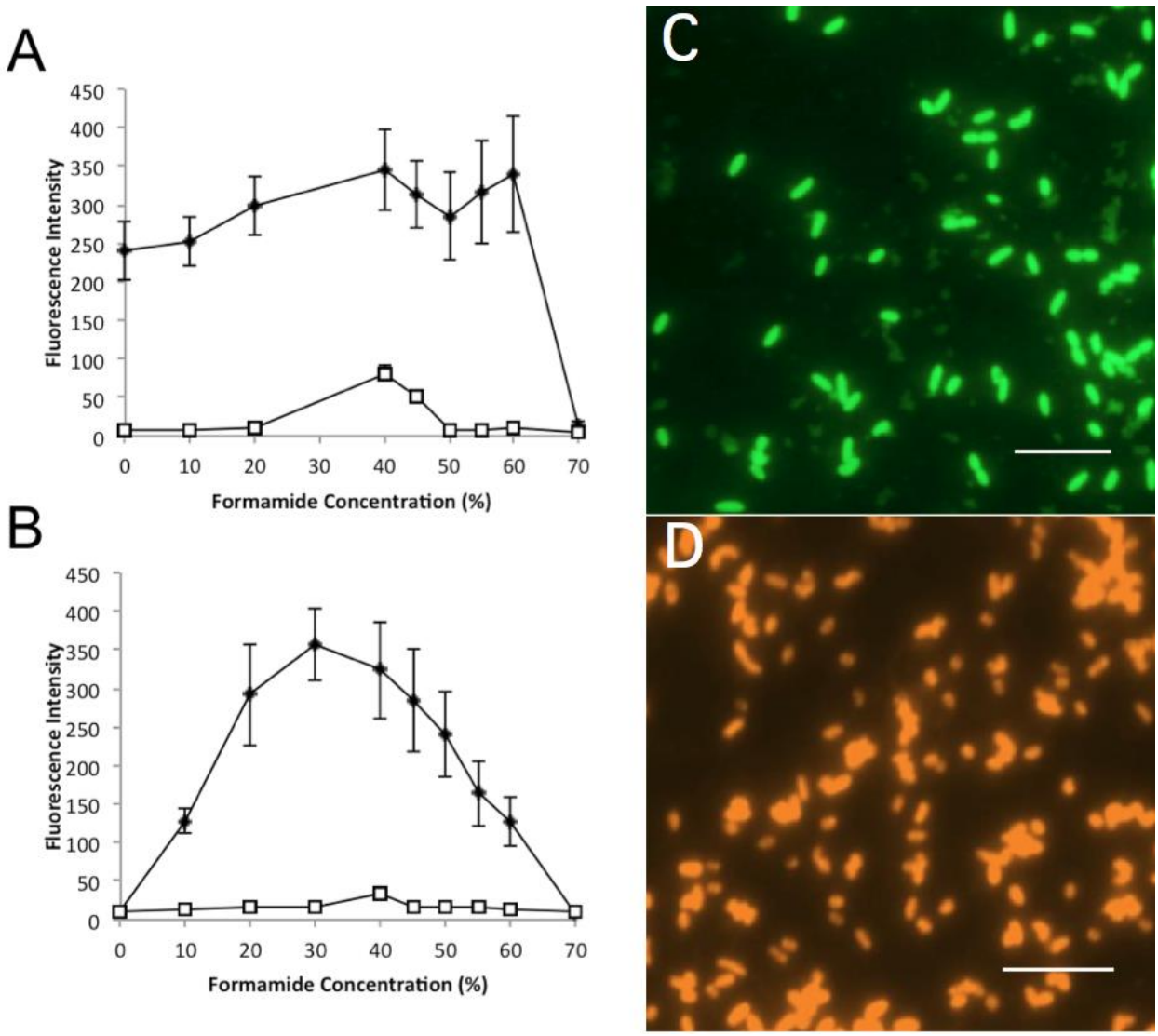

Figure 1. Dissociation profiles of 16S rRNA-targeted probes PCY223 and ALGAR209 for CARD-FISH evaluated against the perfectly-matching (target) strains *P. algicola* and *A. aromaticivorans*, respectively, over a range of formamide concentrations (%). Black diamonds represent hybridization intensities for *P. algicola* (A) or *A. aromaticivorans* (B) with their respective probes. White squares correspond to hybridization intensities for these same organisms, but using the NON338 probe. These probes were labeled with CY3 in all experiments.

Each data point represents the average fluorescence intensity value ± standard deviation up to eight randomly-selected fields of view. In some cases the standard deviation is smaller than the symbol. Images of the target cells were captured from hybridisations performed with probe PCY223 (C) or ALGAR209 (D) using the formamide concentration determined optimal for each probe – 55% for PCY223 and 40% for ALGAR209. The competitor probe c1ALGAR20 was included together in hybridisations with ALGAR209. Bar, 4 μm.

Detection and response of *P. algicola* and *A. aromaticivorans* in micro-algal cultures enriched with phenanthrene

In the present study the aim was to visualize, for the first time, the presence of these OHCB on the surface (phycosphere) of micro-algae. The localization of the hydrocarbonoclastic bacterial in relation to their micro-algal hosts and during petroleum hydrocarbon enrichment may be important factors governing the relationship of these organisms. The CARD-FISH probes designed and optimized for the detection of *P. algicola* and *A. aromaticivorans* were used to directly visualize these bacteria in association with marine eukaryotic phytoplankton (micro-algae), which is likely their natural biotope in the ocean. For this, micro-algal cultures *S. costatum* and *L. polyedrum* were used as they represent the original source from which *P. algicola* and *A. aromaticivorans*, respectively, were isolated (Gutierrez *et al*., 2012a; Gutierrez *et al*., 2013). Subsamples taken prior to and during enrichment were analysed in order to examine the response of these bacteria to phenanthrene, which was shown as a preferred carbon source for these obligate hydrocarbon-degrading bacteria (Gutierrez *et al*., 2012a; Gutierrez *et al*., 2013) and considered a model hydrocarbon to enrich for these organisms.

In the enrichment employing *S. costatum*, subsamples analysed with Cy3-labelled PCY223 revealed an extremely low abundance of *P. algicola*, with either none or one to three cells detected in any of the fields of view analysed (Figure 2A). By day 5, the abundance of *P. algicola* cells quantified with probe PCY223 had increased as a result of enrichment with

phenanthrene, with *P. algicola* signals observed attached with, and detached from, the diatom (Figure 2B). Hybridisations performed with subsamples taken at day 8 (results not shown) did not noticeably differ to those examined at day 5. In the enrichment employing *L. polyedrum*, subsamples analysed with Cy3-labelled ALGAR209 also revealed a very low abundance of *A. aromaticivorans* cells, none of which were observed attached to dinoflagellate cells in any of the fields of view analysed (Figure 2C). By day 3, the abundance of these bacteria, as quantified with probe ALGAR209, was observed to have moderately increased as a result of enrichment with phenanthrene (results not shown), and then markedly increased by day 8 (Figure 2D). Signals for *A. aromaticivorans* cells were observed attached to cells of *L. polyedrum* (Figure 2D), and in some fields of view some cells were found associated with clusters of other (DAPI-stained) bacteria. After incubation with phenanthrene, cells of the hydrocarbonoclastic bacteria in both the *L. polyedrum* and *S. costatum* cultures appeared to be arranged in clusters (or arranged in streaks), where previously (on day 0) they were present as unattached single cells, sometimes appearing near micro-algal cells. These micro-aggregate (<5 – 500 μm) arrangements of bacteria and micro-algae have also been reported in the literature (Simon *et al*., 2002). The distribution of these OHCB appears uneven among the rest of the DAPI-stained bacterial community, suggesting that these bacteria are possibly either aggregating towards a hydrocarbon source that may be adsorbed onto the micro-algal cells and/or are being held together by some transparent extracellular polymer (as proposed by Thornton, 2002).

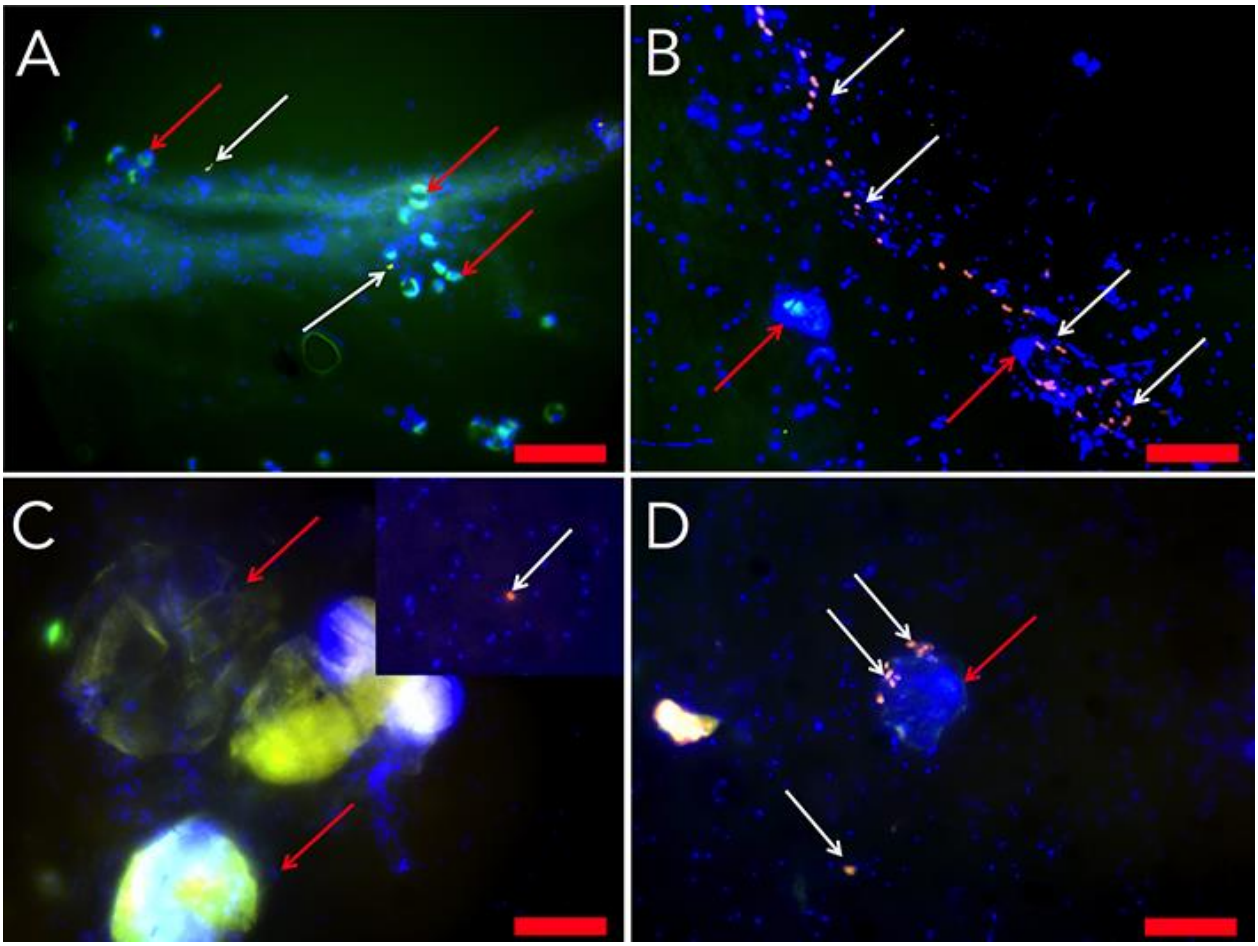

Figure 2. Hybridisation of samples from a culture of *Skeletonema costatum* (A, B) or *Lingulodinium polyedrum* (C, D) enriched with phenanthrene using, respectively, probe PCY223 targeting *P. algicola* or probe ALGAR209 (and competitor probe cALGAR209) targeting *A. aromaticivorans* (orange) among cells of the diatom/dinoflagellate and other bacteria (blue). Samples were taken for analysis on day 0 (A, C), day 5 (B) or day 8 (D). Cells of *P. algicola* and *A. aromaticivorans* (ca. 1 μm size) are indicated with white arrows in the panels, whereas cells of *S. costatum* cells (ca. 10 μm) or *Lingulodinium polyedrum* (ca. 30 μm) are indicated with red arrows. Bar, 20 μm.

Overall, in both enrichments with the *S. costatum* and *L. polyedrum* cultures, most fields of view rarely revealed any signals for, respectively, *P. algicola* and *A. aromaticivorans*. This indicates that these bacteria are in very low abundance to the total bacterial composition

associated with these micro-algae. The observed attachment of some cells for both types of bacteria to their micro-algal host cells infers that they may share more than just a physical attachment, as reported for other types of bacteria living associated with micro-algae. The interaction of micro-algae with bacteria in the ocean is believed to play an important role to their ecological success (Amin *et al*., 2012; Amin *et al*., 2015; Buchan *et al*., 2014). Algal-associated bacteria have been shown to acquire algal exudates as carbon and energy sources (Bell and Mitchell, 1972; Myklestad, 1995), where the algae have been reported to benefit from bacterial-produced vitamins, trace metals and other nutrients (Kazamia *et al*., 2012; McGenity *et al*., 2012). In a study by Amin et al. (2009), the authors reported a mutual sharing of iron and fixed carbon between several species of phytoplankton and bacteria, including members comprising the hydrocarbon-degrading genus *Marinobacter*. Taking into account the narrow nutritional spectrum of strains TG408 and DG1253 living associated with micro-algae (Gutierrez *et al*., 2012a; Gutierrez *et al*., 2013), it is possible that they too may co-exist through some form of nutrient-sharing, though further work will be needed to determine this.

Detection and response of *P. algicola* and *A. aromaticivorans* in natural seawater populations of phytoplankton enriched with crude oil

The enrichment and spatial localization of *P. algicola* and *A. aromaticivorans* was investigated in a natural population of phytoplankton from coastal water. Since these obligate hydrocarbonoclastic bacteria were originally isolated and shown to live associated with laboratory cultures of micro-algae (Gutierrez *et al*., 2012a; Gutierrez *et al*., 2013), the application of CARD-FISH employing the new probes (PCY223 and ALGAR209) allowed us to observe these bacteria in natural seawater samples, including associated with phytoplankton cells. This was examined in enrichments with and without crude oil in order to detect for these bacteria under, respectively, perturbed and unperturbed environmental conditions. Samples taken at days 0 (results not shown) and 2 of these enrichment experiments and analysed with probes PCY223

or ALGAR209 rarely revealed signals for *P. algicola* (Figure 3A) or *A. aromaticivorans* (Figure 3C), respectively, but were nonetheless detected in the field sample. In samples taken at day 5, however, cell signals for these bacteria were slightly more abundant, though still rare to find in the many fields of view observed under the microscope (Figure 3B and D). At this time point, cells of *P. algicola* and *A. aromaticivorans* were observed among the free-living bacterial population that was counterstained with DAPI, as well as attached to cells of micro-algae, including cells of *Skeletonema* spp., likely *S. costatum*. It was apparent that the crude oil had enriched for these bacteria, but to heightened levels of cell abundance that were still very low compared to their abundance in the original unperturbed phytoplankton field sample. These results corroborate those from our analysis of these bacteria in laboratory cultures of micro-algae where very few cell signals had been detected, and where the abundance of these bacteria appeared to have only slightly increased after enrichment with phenanthrene.

Although the primary objective of these enrichment experiments was to test the newly designed PCY223 and ALGAR209 probes, and to directly visualize the target organisms (*P. algicola* and *A. aromaticivorans*) with micro-algae, our data offers some insight into the ecology and dynamics of these obligate hydrocarbon-degrading bacteria during exposure to a hydrocarbon. In a previous study, qPCR probes were used to quantify the abundance of *P. algicola* 16S rRNA gene sequences during enrichment of *S. costatum* CCAP 1077/1C – the same strain used in the present study – with crude oil, and showed these genes to have increased by one order of magnitude within eights days of exposure to the oil (Mishamandani *et al*., 2016). This corroborates our CARD-FISH analysis with the PCY223 probe that showed a distinct increase in the abundance of *P. algicola* signals within 8 days exposure to hydrocarbons. The study by Mishamandani *et al*., (2016) further showed that the abundance of *P. algicola* 16S rRNA genes continued to increase, by a further three orders of magnitude, by day 42 of the crude oil enrichment experiment with *S. costatum*.

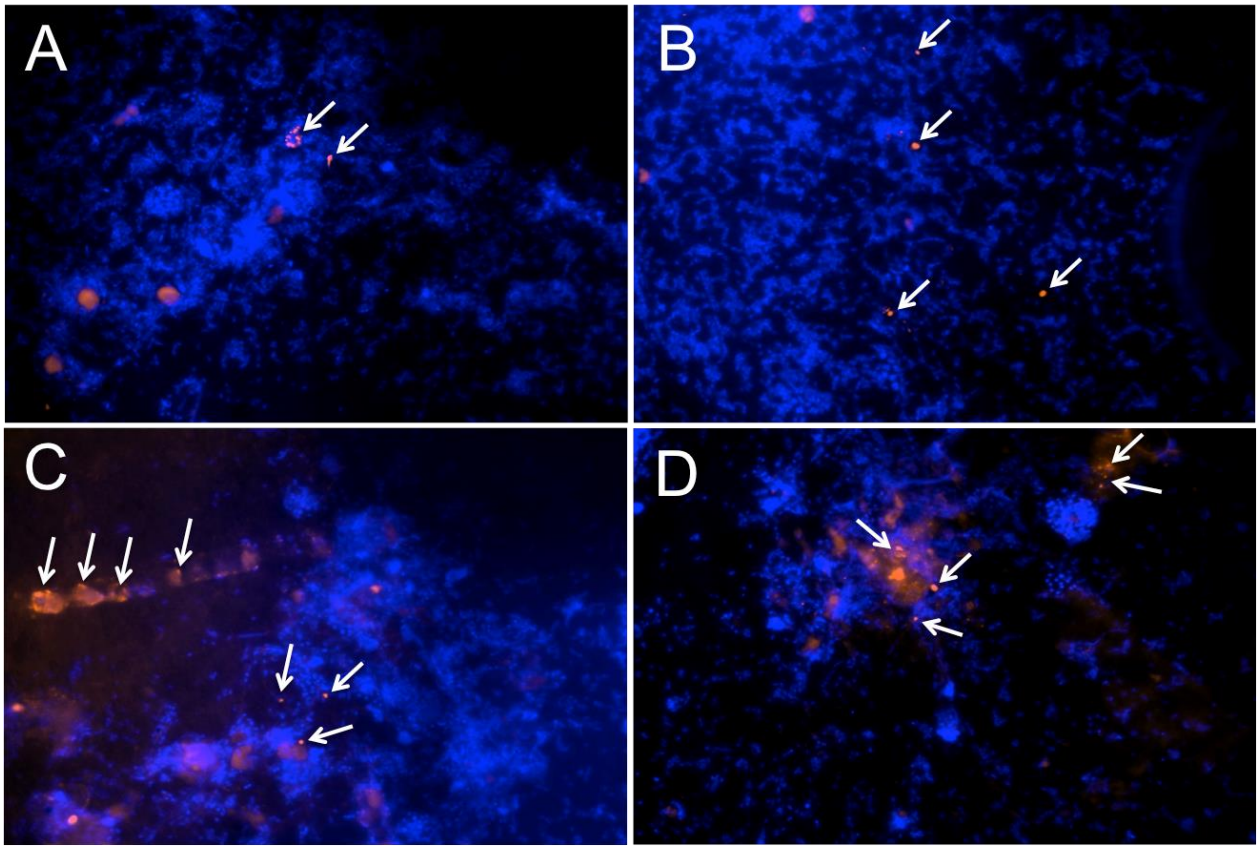

Figure 3. Hybridization of samples from the crude oil enrichment with probe PCY223 at days 2 (A) and 5 (B), and with probe ALGAR209 at days 2 (C) and 5 (D) amongst the entire DAPI-stained microbial population (blue). White arrows indicate cell signals targeted by the respective probe. Small spherical micro-algal cells (orange autofluorescence) are shown in (A) with no apparent associated *P. algicola* cell signals; a *Skeletonema* chain (orange autofluorescence) is observed in (C) with several *A. aromaticivorans* cell signals associated with it.

Based on initial abundance of these bacteria and their response to PAH/crude oil enrichment, it is possible that these types of obligate hydrocarbonoclastic bacteria may contribute to background levels of hydrocarbon biodegradation in the ocean rather than as major responders to oil spills. This is supported by the fact that they are poorly represented in 16S rRNA gene

sequence databases, including datasets from sequencing surveys performed on samples collected at natural oil seeps and oil spill sites (e.g. Deepwater Horizon).

Conclusions

The low abundance of *P. algicola* and *A. aromaticivorans* observed in our phenanthrene enrichment experiments, and the fact that 16S rRNA gene sequences of these bacteria are poorly represented in sequencing surveys in the literature, suggests that they are likely to contribute to the biodegradation of hydrocarbons in the water column at background levels. These organisms have been shown to be associated with a range of other species of diatoms, dinoflagellates and coccolithophores (Gutierrez *et al*., 2013; and unpublished data). It may be inferred that they have eluded detection in sequencing surveys because they occupy a specific biotope in the ocean (i.e., the phycosphere of phytoplankton), which has not been sufficiently explored in this respect, and because these bacteria may comprise the rare biosphere in the ocean. The design and development of 16S rRNA oligonucleotide probes for CARD-FISH provides a useful tool to study the occurrence and ecology of hydrocarbon-degrading bacteria in laboratory cultures of micro-algae and in fresh field samples. These new probes (PCY223 and ALGAL209) also expand the current inventory of FISH probes for targeting hydrocarbon-degrading organisms that will contribute to improving our understanding on the role of these bacteria in the ocean hydrocarbon cycle.

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